



## Standardization of Indian cultivars of *Brassica* spp. for characterization of *Xanthomonas campestris* pv. *campestris* races causing black rot disease of crucifer crops

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### ABSTRACT

*Xanthomonas campestris* pv. *Campestris* (Xcc) causing black rot disease of crucifer crops, is a serious worldwide problem resulting in >50% loss under environmental conditions favorable to the pathogen. For the characterization of *Xanthomonas campestris* pv. *campestris* races to standardize the Indian cultivars of *Brassica* spp. total of 184 representatives lines of the family crucifereae from of five economically important *Brassica* species, i.e. *B. carinata*, *B. juncea*, *B. napus*, *B. oleracea*, and *B. rapa* grown in India were inoculated by 75 strains of Xcc, belonging to three races 1, 4 and 6 in 2012-13 and 2013-14. Disease reaction of these cultivars was compared with the standard differential hosts of crucifer for disease incidence and diseases severity. Indian cultivars of *B. juncea* (Pusa Bold, Pusa Varuna, Pusa Vijay, Pusa Mustard-21, Pusa Mustard-25, Pusa Sag), *B. oleracea* var. *botrytis* (Pusa Sukti), *B. oleracea* var. *capitata* (KGMR) and *B. rapa* (Pusa Swarnima) were at par with the standard differentials of respective *Brassica* species and thus could be suitable to use as alternative differential hosts for characterization of Xcc races. 20 cultivars of *Brassica* spp. grown in India were further validated as differential crucifer host to characterize the races of Xcc on the molecular basis using four set of primers, viz. CAPS (ACS2) CAPS (BTPT) and CAPS (NDPK3) and among them, Br 019305 primer amplified at 906 bp in all cultivars, while primer CAPS (ACS2), CAPS (BTPT) and CAPS (NDPK3) primers were amplified multiple bands. Combined phylogenetic UPGMA study at 75% similarity coefficient, 19/20 accessions were divided into three groups and distinguished resistant and susceptible cultivars against black rot disease.

**Key words:** *Brassica* spp, Differentials, Pathogenic variability, Races, Resistance

Black rot, caused by *Xanthomonas campestris* pv. *campestris* Pamell Dowson (Xcc), is a very destructive disease of crucifers worldwide causing severe damage under favourable environmental conditions (Singh *et al.* 2015). *X. campestris* pv. *Campestris* is also differentiates into pathogenic variants called races. Race characterization of the pathogen is important for development of resistant varieties to control the disease. Since the first recognition of races of *X. campestris* pv. *campestris* was done by Kamoun *et al.* (1992). There is apparent need to define resistance in relation to the race type of the isolate or isolates used. Isolates of *X. campestris* pv. *campestris* were initially separated into five races (0 to 4) based on the reaction of cultivars of *Brassica rapa* and *B. juncea* (Kamoun *et al.* 1990) and later reclassification of the race system into six

races (1 to 6) was made on the basis of the reactions of an extended series of differential *Brassica* genotypes derived in part from the series of Kamoun *et al.* 1992 and Vicente *et al.* 2001. The current race structure is further defined on the basis of a gene-for gene relationship involving the postulated interactions of four matching gene pairs: avirulence (A) genes in the pathogen races and resistance (R) genes in the differential hosts. The currently defined races, only races 1 and 4 seem to be of major importance worldwide in *B. oleracea*, and races 2, 3, 5 and 6 are rare (Vicente *et al.* 2001, Vicente *et al.* 2006). Variation between *X. campestris* pv. *campestris* isolates was recognized and it was generally considered to represent merely a difference in aggressiveness in differential hosts. Differential hosts are the host of particular pathogen, which defined or show differences in pathogenicity. The inheritance of major gene resistance has been studied in the diploid *B. rapa* (A genome), and in the tetraploids *B. carinata* (BC genome) and *B. napus* (AC genome) (Guo *et al.* 1991, Ignatov *et al.* 2000, Vicente *et al.* 2002). A single dominant race-specific gene has been mapped to the A genome in *B. napus* (Vicente *et al.* 2002), and quantitative loci that control resistance to at

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least two of the most prevalent races of *X. campestris* pv. *campestris* have been mapped in a Chinese cabbage accession of *B. rapa* (Soengas *et al.* 2007). Genes present in the *Brassica* A and B genomes could potentially provide durable black rot control, especially if strong race specific genes (matching the most prevalent races) could be combined in a genetic background of race non-specific genes, providing quantitative resistance. Differential hosts of *X. campestris* pv. *campestris* races are required and these are not available in India. These differential cultivars are needed to import from other countries, which is costly and time consuming.

Standardization of the differential hosts of Indian cultivars of *Brassica* spp. for characterization of races of Xcc by comparing disease severity with international standard differential hosts and by molecular approach, will help to develop resistant varieties against the races this pathogen.

## MATERIALS AND METHODS

For race characterization of *X. campestris* pv. *campestris*, it is important to standardize the differential cultivar of crucifers. International standards of differential host of *Brassica* species viz. turnip (*Brassica rapa* var. *rapa*) Just Right Turnip F1 and Seven Top Turnip, Indian mustard [*B. juncea* (L.) Czern and Coss] Florida Broad Leaf, Ethiopian mustard (*B. carinata* L. Braun) PI 199947, rapeseed mustard (*B. napus* L.), Cobra 14R, cauliflower (*B. oleracea* var. *botrytis* L.) Miracle F1 and cabbage (*B. oleracea* var. *capitata* L.) Wirosa F1 were used under this study. The seeds of these differential lines were obtained from University of Warwick, UK and from Otis S. Twilley Seed Co. Inc. 121 Gary Rd, Hodges SC 29653-9168 [*B. juncea*, *B. rapa* var. *rapa* (EC732033 to EC732035)].

A total of 184 accession of *Brassica* spp. such as *B. carinata*, *B. juncea*, *B. napus*, *B. oleracea*, and *B. rapa* were taken for screening against 75 strains of *X. campestris* pv. *campestris* and out of these, 19 accessions of cultivars of cruciferae family comprising 5 economically important *Brassica* species, viz. *B. campestris*, *B. carinata*, *B. juncea*, *B. napus*, *B. oleracea*, and *B. rapa* were taken for comparative study with international standard differentials for characterization of races. The seeds of these cultivars/lines were obtained from Division of Genetics and Division of Vegetable Sciences, IARI, New Delhi. They were grown at IARI Farm, New Delhi. The pathogenic variability was studied during winters (November to March) for two consecutive years, viz. 2012–13 and 2013–14 following the standard procedure of crop cultivation.

Seventy five isolates were isolated from 18 states of India (Rathaur *et al.* 2015) belonging to race 1, 4 and 6 of *X. campestris* pv. *Campestris* were grown on yeast extract glucose calcium carbonate agar (YGCA) medium (yeast extract: 10 g, of D-glucose anhydrous: 10 g, of calcium carbonate: 20 g and of agar powder: 20 g, double distilled water 1 000 ml) at 28°C for 48 h and then the bacterial growth was scraped from the Petri plates, and the inoculum was maintained adjusted to 0.1 optical densities (at 600 nm)

bysteric distilled water (Schaad *et al.* 2001, Singh *et al.* 2014).

This experiment was carried out between October to March with temperature 19.8–32.7°C and humidity 83–92%. *Brassica* leaves were inoculated by 75 isolates of Xcc by cutting a small cut, near the margins, with scissor, 35 days after sowing of oil seed crops, turnip and radish and 25 days after transplanting of cole crops. Totally, 10 cuts were given to each leaf by dipping the scissor in the bacterial suspension. The three young leaves of each cultivar were inoculated. Also provide the details of humidity and temperature control during the experimentation.

These verity of symptoms was assessed 21 days after inoculation by using a 0–9 scale based on the relative lesion sizes: 0 = no symptoms, 1=small necrosis or chlorosis surrounding the infection point, 3=typical small V-shaped lesion with black veins, 5=typical lesion half way to the middle vein, 7=typical lesion progressing to the middle vein and 9=lesion reaching up to midrib (Vicente *et al.* 2001). The data generated on disease severity were statistically analyzed.

The DNA was isolated from the freeze-dried leaf tissue of *Brassica* lines using cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). DNA was purified as per the protocol with minor modifications. 2.0 µl of RNase (10 mg/ml) was added in the extracted 200 µl DNA in micro-centrifuge tube and incubated at 37°C for 1 h. The purified DNA was quantified by running 2 µl of each DNA sample on 0.8% agarose gel along with the uncut lambda DNA (100 ng/ µl). The final concentration was made of 25–50 ng/ µl for use in PCR analysis.

Four primers, viz. Br 019305, CAPS (ACS2) CAPS (BTPT) and CAPS (NDPK3) were used as designed by Doullah *et al.* (2011). About 25.0 µl of PCR reaction mixture containing 5 × Taq buffer (5.0 µl), 10 mM dNTPs (0.5 µl), 25 mM MgCl<sub>2</sub> (1.5 µl), 10 µM forward and reverse primers (0.5 µl each), Taq polymerase (0.24 µl), molecular grade water and 1.0 µl DNA (100 ng) was used for PCR amplification. PCR was carried out under the following conditions using a Gradient thermocycler (C-1000TM, BIORAD): 95°C for two min followed by 30 cycles of 95°C for 30 sec, annealing temperature and duration was varied according to gene, 72°C for 1 min and terminated by a final elongation at 72°C for 5 min (Doullah *et al.* 2001). The PCR product was mixed with 1.0 µl of loading dye. Electrophoresis was carried out using 1.2% agarose gel, prepared in 1 × Tris-acetate-EDTA (TAE) buffer containing ethidium bromide (0.5 µg/ml). Electrophoresis was carried out at 60V for 1.5 hr, visualised on a gel documentation system under UV light (300 nm) and photographed using image lab version 2.0.1 software for gel analysis. The data from gel profiles were combined together to generate a similarity matrix by using the SIMQUAL module of NTSYSpc 2.02e. The output data are graphically presented as a phylogenetic tree. All the *Brassica* spp. could be clustered into 3 groups at 75% similarity coefficient to separate resistant and susceptible cultivars of *Brassica* spp.

Table 1 Primer used in molecular characterization of Indian and Standard differential cultivars for the race characterization of *X. campestris* pv. *campestris*

Name	Sequences of primer (5'—3')	Product size (bp)	Annealing temperature and duration	References
Bra019305	F: GAGCCGTTGAGACTCCCTT R: CCACTTCTCCACCGGCTTA	906 bp	52°C	“
CAPS 1(ACS2)	F: AGCTACATGCAACAGCCATG R: AGTCGTTGTCTTCTTCCTCG	Multiple bands	52°C	“
CAPS 2 (BTPT)	F: AGATCTCCCACGATGCAGAG R: AGTAAGTCAGCAAAGAGAAC	Multiple bands	55°C	“
CAPS 2 (NDPK3)	F: GGTCTTCTCTCAGTTTCACT R: AGTTATCGCCATAGATCCAC	Multiple bands	54°C	“

## RESULTS AND DISCUSSION

### Screening for resistance to *X. campestris* pv. *Campestris* races

The results of the 184 cultivars of *Brassica* spp. (*B. carinata*, *B. juncea*, *B. napus*, *B. oleracea*, and *B. rapa* making the subset from *Brassica* spp.) were provided the details of accessions of 41 cultivars were given in supplementary Table 1 while remaining were lines. Out of 184 cultivars, maximum 127 cultivars of cauliflower followed by 26 of Indian mustard and 23 of Ethiopian mustard. 71.1 % of cultivars showed susceptible reaction and 25.5% showed resistant reaction against black rot disease caused by 75 isolates of *X. campestris* pv. *campestris*. Variation in disease severity on among *X. campestris* pv. *campestris* races significantly. Only *B. juncea* (AB genome) cultivars such as Pusa Bold, Pusa Varuna, Pusa Vijay, Pusa Mustard-21, Pusa Mustard-25 and Pusa Sag showed highly resistant against all the three races (Race 1, 4 and 6) and it was potential race-nonspecific resistance. One accession of *B. oleracea* var. *capitata*, Pusa Agetiwas resistant to race 1 and 4 but showed susceptible reaction against race 6 of Xcc (Xcc-C278). One cultivar of *B. carinata* (BC genome) Pusa Swarnim (IGC-01) and *B. rapa* (A genome) Pusa Swarnima showed partial resistant. *B. oleracea* var. *botrytis* tested was susceptible to the three races. Race 4 was present in a high proportion of the *B. napus* and *B. rapa* accessions tested. Generally, the rape cultivars (e.g. GSL-1,2 and 5, PAC-401) showed a low pathogenic to race 4. Out of 127 cultivars of cauliflower, 120 showed susceptible against race 1, 4, and 6 and 7 accessions were showed partial resistant. Cultivars of *B. juncea* and *B. carinata* (BC genome) were highly variable. One accession (Pusa Swarnim IGC-01) showed partial resistance to the two races (potential race-nonspecific resistance). The identification of potentially race-nonspecific resistance in the A and the B genomes was possibly the most interesting finding of this study. This resistance was distinguished from that conferred by R1 or R1 and R4, particularly by the response to race 6. Similar race nonspecific resistance was reported with bacterial pathogens of leguminous hosts (*Pseudomonas syringae* pv. *Phaseolicola* in beans and *P. syringae* pv. *Pisi* in peas). In

these cases, race-nonspecific resistance was due to recessive genes (Elvira-Recuenco *et al.* 2000, Teverson *et al.* 1991). It has been suggested that, for peas and beans, durable resistance might be achieved by the combination of race-specific resistance and race-nonspecific resistance (Taylor *et al.* 1996).

### Standardization of Indian differential hosts for race characterization

To identify the differential host for characterization of *X. campestris* pv. *campestris* races from Indian cultivars of *Brassica* spp. disease severity caused by this pathogen was compared between international standard differentials and Indian cultivars/lines of *Brassica*. Out of 19 cultivars of *Brassica* spp. such as *B. juncea*, *B. carinata*, *B. oleracea* var. *capitata*, *B. oleracea* var. *botrytis* and *B. rapa*, showed no significant variation in disease severity between international standard differentials and Indian cultivars/lines. In *B. juncea*, international standard differentials were Florida Broad Leaf and its disease severity was similar with six Indian accessions, i.e. Pusa Bold, Pusa Varuna, Pusa Vijay, Pusa Mustard 21 and Pusa Mustard 25 and Pusa Sag. *B. oleracea* var. *capitata*, standard differential was Wirosa F1, which was similar to *B. oleracea* (KGMR), *B. oleracea* var. *botrytis*, Miracle F1 was similar to Pusa Sukti and in *B. rapa*, Seven Top Turnip was similar with Pusa Swarnima (Table 2). However remaining three standard differential cultivars like accession PI199947 (*B. carinata*), Cobra 14 R (*B. napus*) and Just Right Turnip (*B. rapa*) varied in disease severity significantly and they cannot be used as differential hosts for characterization of races. A set of seven differential hosts for characterization of races of *X. campestris* pv. *Campestris* are earlier used by Taylor *et al.* (1996) and Vicente (2001), which was corroborative to our results.

### Molecular characterization

In this study molecular based screening of *Brassica* cultivar has been done. Four primers were screened and used in this study and Br 019305 primer and CAPS (NDPK3) amplified in same bands in all *Brassica* spp. (Table 1). Other primers were showing multiple bands, banding pattern were same at species level in *B. oleracea*, *B. rapa* and *B.*

Table 2 Comparative black rot disease severity caused by *X. campestris* pv. *campestris* between international standard differential cultivars and Indian cultivars of *Brassica* for race characterization

Crop Name	Indian cultivars	Disease severity (0- 9 scale)	International standard differential cultivars	Disease severity (0- 9 scale)
Indian mustard ( <i>B. juncea</i> )	Pusa Bold	0 <sup>f</sup>	Florida Broad Leaf Mustard	0 <sup>f</sup>
	Varuna	0 <sup>f</sup>		
	Pusa Vijay	0 <sup>f</sup>		
	Pusa Mustard 21	0 <sup>f</sup>		
	Pusa Mustard 25	0 <sup>f</sup>		
	Pusa Sag	0 <sup>f</sup>		
Ethopian mustard ( <i>B. carinata</i> )	Pusa Swarnim(IGC-01)	3.4 <sup>bcd</sup>	PI 199947	1.0 <sup>ef</sup>
	Pusa Aditya(NPC-9)	4.2 <sup>ab</sup>		
	HC-2	4.2 <sup>ab</sup>		
	Kiran	4.7 <sup>ab</sup>		
Rapeseed ( <i>B. napus</i> )	GSL-1	4.6 <sup>ab</sup>	Cobra 14 R	2.4 <sup>cde</sup>
	GSL-2	4.8 <sup>ab</sup>		
	GSC-5	4.9 <sup>ab</sup>		
	PAC-401	4.8 <sup>ab</sup>		
Cauliflower ( <i>B. oleracea</i> var. <i>botrytis</i> )	Pusa Sharad	5.2 <sup>a</sup>	Miracle F1	4.9 <sup>ab</sup>
	Pusa Sukti	4.5 <sup>ab</sup>		
Cabbage ( <i>B. oleracea</i> var. <i>capitata</i> )	Pusa Ageti	2.0 <sup>ab</sup>	Wirosa F1	4.0 <sup>ab</sup>
	KGMR	4.4 <sup>ab</sup>		
Turnip ( <i>B. rapa</i> )	Pusa Swarnima	3.7 <sup>ab</sup>	Seven Top Turnip	2.3 <sup>cde</sup>
	Pusa Swati	4.5 <sup>ab</sup>	Just Right Turnip F1	2.13 <sup>de</sup>

*juncea* cultivars. *B. carinata* and *B. napus* were showed. In case of CAPS (ACS2) primer, *B. oleracea* cultivars showed same bands but cultivars of *B. rapa* (Seven Top Turnip and Pusa Swarnima) also showed same banding pattern as *B. juncea*, which was resistant to black rot disease. Other two *B. rapa* cultivars had showed different banding pattern from Seven Top Turnip and Pusa Swarnima. *B. juncea* cultivars showed different unique pattern of bands. In CAPS (BTPT) primer, all cultivars of *Brassica* spp. amplified at same level of band pattern. *B. juncea* and some cultivar of *B. napus* amplified at different level band pattern.

Based on data generated by using primers combined phylogenetic was prepared at 75% similarity coefficient. All cultivars of *Brassica* clustered into three groups. This grouping indicates genome bases similarity (Fig 1). In first group, *B. oleracea* (C genome), *B. carinata* (BC genome) and *B. napus* (AC genome) cultivars were grouped together but in different clades. All *B. oleracea* cultivars (KGMR, Miracle F1, Pusa Sukti) grouped into same clade with standardized cultivar Wirosa F1. *B. carinata* (BC genome) cultivars (Pusa Swarnim (IGC-01), Pusa Aditya (NPC-9), HC-2, Kiran) and *B. napus* (AC genome) (GSL-1, GSL-2, GSL-5, PAC- 401) cultivars are grouped in a same cluster with their respective standard cultivars, i.e. PI 199947 and Cobra 14 R. Two cultivars, Just Right Turnip and Pusa Swati of *B. rapa* (A genome) formed separate cluster under Group 2, while Seven Top Turnip and Pusa Swarnima of *B. rapa* and Pusa Vijay of *B. juncea* grouped in same group. Most of *B. juncea* cultivars (Pusa Bold, Pusa Varuna, Pusa Mustard 21, Pusa Mustard 25 and Florida Broad Leaf) grouped into same clade except Pusa Vijay and they clustered

into Group 3. Cultivars of in this group were found resistant to races 1, 4 and 6 of *X. campestris* pv. *campestris*. Phylogenetic result was similar with disease severity result because on the basis of disease severity, shortlisted cultivars were grouped in same way in dendrogram also. The primer used in this study was earlier reported by Doullah *et al.* (2011). Although the most useful sources of resistance are present in the A and B genomes (*B. rapa* and *B. nigra*) and absent in C genome (*B. oleracea*) (Vicente *et al.* 2001, 2002; Westman 1998). Resistance is inherited either as a dominant or recessive trait, which depends on genetic background of cultivars as shown by resistant gene to race 3, which is dominant in DH BOH 85c background and recessive in Badger inbred-16 background (Camargo *et al.* 1995).

Total 184 *Brassica* cultivars were used for standardize the suitable cultivars for race identification of *X. campestris* pv. *campestris*. Indian cultivars of *Brassica* spp., Mustard 21 and Pusa Mustard 25 and Pusa Sag of *B. juncea*, KGMR of *B. oleracea* var. *capitata*, Pusa Sukti of *B. oleracea* var. *botrytis* and Pusa Swarnima of *B. rapa* may be used as differential hosts for race characterization of *X. campestris* pv. *campestris*. However, Indian cultivars of *B. carinata*, *B. napus* and *B. rapa* (only Just Right Turnip) did not show similarity in disease severity with international standard of differential hosts and it is required more further study for screening of cultivars. By molecular characterization, differentials cultivars were showing species specific results. This study will be helpful for the development of the differential cultivars for race characterization of *X. campestris* pv. *Campestris* races.

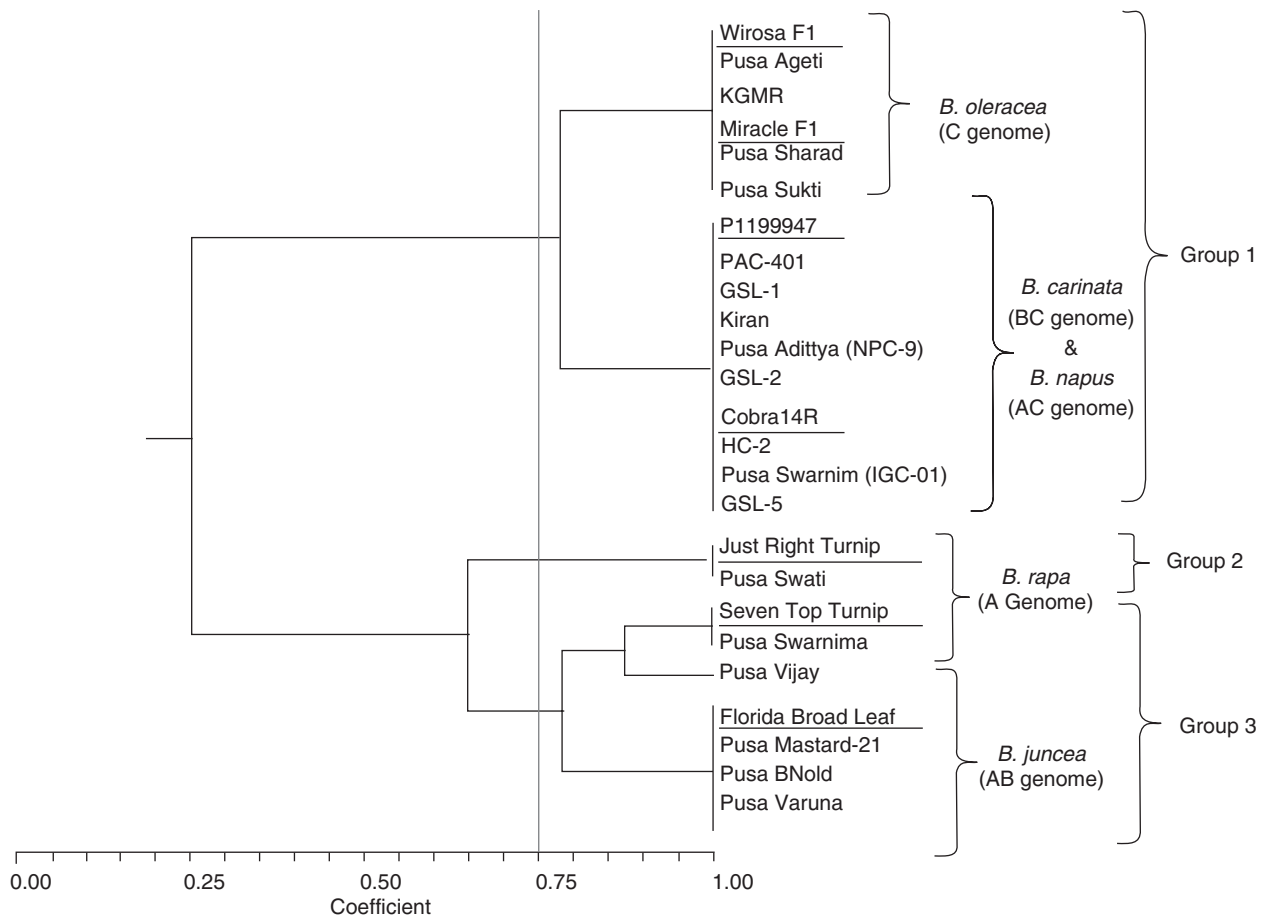


Fig 1 Cluster analysis of *Brassica* cultivars, shortlisted from disease severity result and standard differential cultivars (underlined). Dendrogram was generated using Jaccard's coefficient

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